Biochem. J. (1986) 240, 171–179 (Printed in Great Britain)

Rat heparins

A study of the relative sizes and antithrombin-binding characteristics of heparin proteoglycans, chains and depolymerization products from rat adipose tissue, heart, lungs, peritoneal cavity and skin

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^{35}S-labelled heparins were recovered from adipose tissue, hearts, lungs, peritoneal cavities and skins of rats given $H_2{}^{35}SO_4$. Their purification involved incubation with Pronase, precipitation with cetylpyridinium chloride in 1.0 M-NaCl, gradient elution from DEAE-Sephacel and incubation with chondroitinase ABC. Each product was divided into proteoglycan and 'depolymerization products' fractions by gel filtration on Bio-Gel A-15m. Heparin chains were released from a portion of each proteoglycan fraction by β-elimination with NaOH. Proteoglycans, chains and depolymerization products were separated by gradient elution from a column of antithrombin–agarose into fractions with no affinity, low affinity and high affinity for antithrombin. The relative sizes of the products were determined by gel filtration on columns of Bio-Gel A-50m, A-15m, A-1.5m and A-0.5m. Skin was the major source of heparin and contained the largest proteoglycans and the lowest proportion of depolymerization products. Lungs contained the smallest proteoglycans, the smallest depolymerization products and the highest proportion of depolymerization products. The highest proportions of proteoglycans, chains and depolymerization products with high affinity for antithrombin were found in adipose tissue. The lowest proportions of each of these fractions were found in the peritoneal cavity. The data suggest that there was relatively little biosynthesis of sites with high affinity for antithrombin in peritoneal-cavity mast cells and that heparin catabolism was most active in lungs. Each source of heparin was unique with respect to both biosynthesis and subsequent breakdown of its proteoglycans.

INTRODUCTION

A heparin proteoglycan from rat skin has been used in various studies since 1971 (Horner, 1971, 1972; Robinson *et al.*, 1978; Young & Horner, 1979; Horner & Young, 1982). Skin is the only feasible source of adequate amounts of rat heparin for chemical characterization. In the present study, relatively small amounts of ^{35}S-labelled rat heparins have been extracted and purified from other tissues and from washings of the peritoneal cavity. These heparins were fractionated into proteoglycan and depolymerization product fractions by gel filtration and further fractionated by affinity chromatography on antithrombin–agarose. The fractions obtained by the latter procedure were then gel-filtered to determine their relative sizes. The data for heparins from the various sources differ markedly, indicating that heparin metabolism in the rat varies extensively from one tissue to another.

EXPERIMENTAL

Materials and methods

Dextran sulphate (Pharmacia) was purified by precipitation as the cetylpyridinium complex in 3.0 M-NaCl at 40 °C (Scott, 1960). Hyflo-Supercel (Johns Manville) was added before filtration in a coarse-grade sintered-glass funnel. After washing with a 0.1% solution of cetylpyridinium chloride (CPC) in 3.0 M-NaCl at 35–40 °C, the filter cake was transferred to a centrifuge bottle and washed several times with aq. 70% (v/v) ethanol and then with ethanol to dissociate the cetylpyridinium complex and remove the CPC. Dextran sulphate was then dissolved in 1.0 M-NaCl, separated from Hyflo-Supercel by filtration through sintered glass and precipitated as the sodium salt by adding ethanol (3 vol.). The product was washed, first with 75% ethanol, then with ethanol, and dried under reduced pressure at ambient temperature.

Antithrombin–agarose was prepared as described by Horner & Young (1982), except that Bio-Gel A-50m was substituted for Sepharose 4B.

Eight male Wistar rats (average weight 317 g) were each given 2.9 mCi of $H_2{}^{35}SO_4$ in 1 ml of 0.15 M-NaCl by intraperitoneal injection. Their food was then removed, and they were killed 18 h later by anaesthesia with diethyl ether.

Peritoneal lavage was done with 0.15 M-NaCl/0.002 M-EDTA, adjusted to pH 7.4 with NaOH, warmed to 37 °C. The washings were cooled in ice and NaCl was added to a concentration of 1 M. Ethanol (3 vol.) was then added. After standing at 4 °C overnight, a precipitate was recovered by centrifuging (4800 *g* at 0 °C for 2 h), washed with ethanol and dried under reduced pressure at ambient temperature.

Skins were frozen in liquid N_2, placed between plastic-coated domestic food-chopping boards and fragmented by striking the upper board with a hammer.

Abbreviation used: CPC, cetylpyridinium chloride.

Skin pieces were extracted with acetone and light petroleum (b.p. 30–60 °C) in a Soxhlet apparatus and dried in a fume hood at ambient temperature.

Epididymal fat, hearts and lungs were washed in ice-cold 0.15 M-NaCl, and homogenized in acetone, with a Polytron homogenizer (Brinkmann). The solids were recovered by centrifugation (2000 *g* for 30 min at 0 °C), washed with acetone and light petroleum, and dried under reduced pressure at ambient temperature.

Isolation of pure heparins

The procedures described below apply to all heparin sources except skin, which was worked up as described elsewhere (Young & Horner, 1979). Samples of skin heparins were run through the same columns to which the entire products from other sources were applied.

Digestion with Pronase. Each solvent-extracted heparin source was homogenized with the Polytron in 0.1 M-Tris/0.01 M-$CaCl_2$/0.02% chlorhexidine acetate (Ayerst), adjusted to pH 8.0 with HCl; 50 mg of Pronase (Calbiochem) was added to each digest initially and again after 24 h. The final volume of each digestion mixture was approx. 56 ml. The total incubation period, in a shaker bath at 40 °C, was 48 h. NaCl was then added to a concentration of 2.0 M, and 1 h later dextran sulphate was added (0.1 mg/ml). These mixtures were left shaking at 40 °C for several hours and then at ambient temperature overnight.

Precipitation with cetylpyridinium chloride in 1.0 M-NaCl. Each digestion mixture was centrifuged (17000 *g* at ambient temperature for 30 min) to sediment insoluble material, which was washed with 2.0 M-NaCl and re-centrifuged. Each combined supernatant and washing was warmed to 40 °C and mixed with an equal volume of warm 1% (w/v) CPC in water. ^{35}S-labelled glycosaminoglycans were recovered from their cetylpyridinium complexes by using centrifugal filter assemblies (Young & Horner, 1979).

DEAE-Sephacel chromatography. Each CPC-treated product was dissolved in 0.2 M-NaCl, adjusted to pH 2.5 with HCl, and pumped into a column of DEAE-Sephacel (15 cm × 1.6 cm). A linear gradient of 0.2–1.6 M-NaCl at pH 2.5, total volume 320 ml, was then pumped at a rate of 20 ml/h at 4 °C; 5 ml fractions were collected into tubes containing 2 M-sodium acetate (100 μl/tube). The radioactivity of a sample from each fraction was determined by scintillation counting in PCS (Amersham). Fractions containing ^{35}S-labelled heparin (see the Results section and Fig. 1) were combined. Heparin was recovered by precipitation with ethanol.

Digestion with chondroitinase ABC

Treatment of samples of the heparin products from DEAE-Sephacel with HNO_2 at pH 1.5, followed by gel chromatography on Sephadex G-25 (Shively & Conrad, 1976), showed that each product was contaminated with HNO_2-resistant ^{35}S-labelled glycosaminoglycans. The remainder of each product was digested overnight with 1 unit of chondroitinase ABC (Sigma) (Saito *et al.*, 1968) and passed through a column of Sephadex G-50. ^{35}S-labelled heparins, eluted in the void volume, were precipitated with ethanol. The precipitates were gently washed with cold (4 °C) aq. 80% ethanol to remove traces of NaCl, because strong electrolytes suppress the development of metachromasia when heparin binds to Azure A (Jaques & Bell, 1959). The dried products were dissolved in water and heparin mass was determined colorimetrically with Azure A (Lam *et al.*, 1976). Samples were treated with HNO_2 at pH 1.5: no HNO_2-resistant ^{35}S-labelled glycosaminoglycans were detected. Heparin was recovered by freeze-drying the remainder of each aqueous solution.

Gel filtration with Bio-Gel A-15m

Each purified heparin was dissolved in 1.2 M-NaCl/0.05 M-Tris/HCl, pH 8.5 (250 μl), and pumped through a column of Bio-Gel A-15m (25.5 cm × 1.0 cm) at a flow rate of 2 ml/h; 0.6 ml fractions were collected and radioactivity was determined. In most cases this fractionation gave two peaks: a proteoglycan fraction was eluted before a peak of material which was designated 'depolymerization products' (exceptions to this are discussed in the Results section). The products were recovered by precipitation with ethanol.

Gel filtration with Bio-Gels A-50m, A-1.5m and A-0.5m

In addition to Bio-Gel A-15m, columns (25.5 cm × 1.0 cm) of all these gels were used to compare the sizes of various heparin fractions; 0.6 ml fractions were collected at a flow rate of 2 ml/h.

For comparative purposes, 1 mg samples of commercial pig mucosal heparin were run through these columns. Samples (5 μl) were taken from each fraction to determine their relative heparin contents by metachromasia with Azure A (as quantitative data were not required, no corrections were made for the presence of NaCl). The total volume of each column was determined with ^3H_2O. The void volume of each type of Bio-Gel A except A-50m was determined with rat skin ^{35}S-labelled heparin proteoglycan.

Preparation of heparin chains

A portion of each proteoglycan fraction was treated with NaOH to release heparin chains by β-elimination, as described previously (Horner & Young, 1982).

Fractionation of heparins on antithrombin–agarose

Heparin proteoglycans, chains and depolymerization products were fractionated on an antithrombin–agarose column. The gradient elution protocol has been described in detail previously (Horner & Young, 1982). Briefly, heparin eluted isocratically in 0.14 M-NaCl is designated 'no affinity' (NA) heparin; heparin eluted in a subsequent shallow gradient of 0.14–0.40 M-NaCl is designated 'low affinity' (LA) heparin; and heparin eluted in a final steep gradient of 0.40–3.0 M-NaCl is designated 'high affinity' (HA) heparin. This system was again used in the present work, but the formation of reproducible gradients was greatly facilitated by using an LKB Ultrograd gradient mixer rather than the manually controlled equipment used previously. The Ultrograd mixed varying proportions of solutions containing 0.14M-NaCl and 3.0 M-NaCl, each of which also contained 0.003 M-$CaCl_2$ and 0.01 M-Hepes and was adjusted with NaOH to pH 7.3 at ambient temperature. A constant flow rate of approx. 10 ml/h was maintained; 1.5 ml fractions were collected, and samples were taken for scintillation counting. One column of antithrombin–agarose (10 cm × 1.6 cm) was used through-

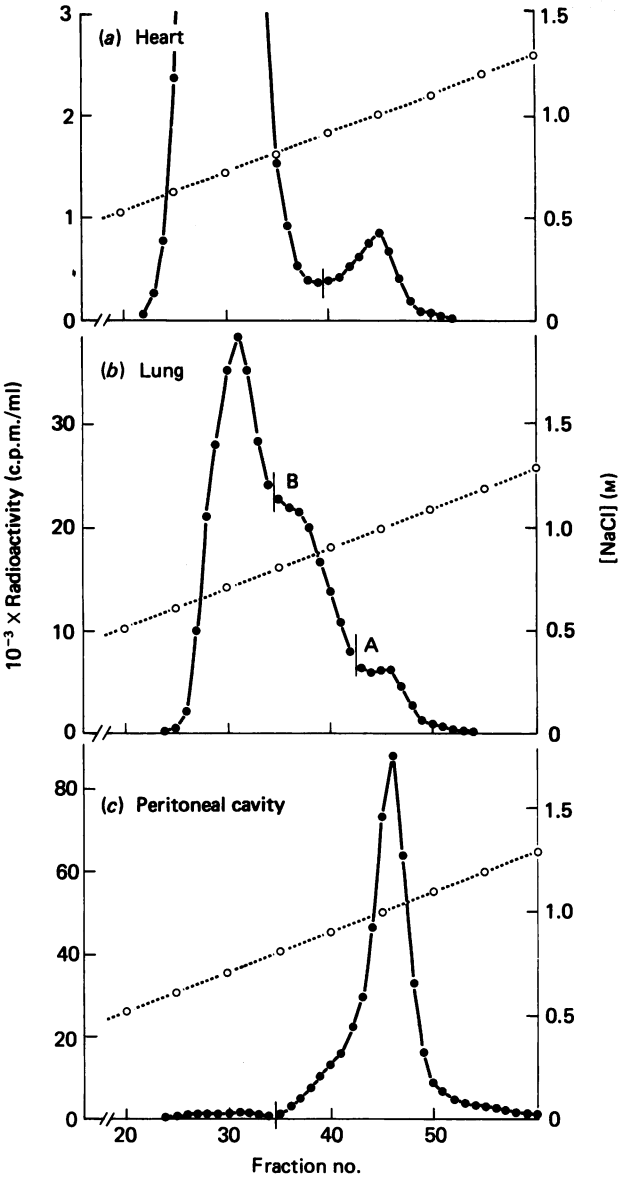

Fig. 1. Elution of ^{35}S-labelled glycosaminoglycans from DEAE-Sephacel with a linear concentration gradient of NaCl, at pH 2.5

The three panels show representative fractionations of products recovered by precipitation with CPC after digestion with Pronase. These are the products from (*a*) heart, (*b*) lung and (*c*) peritoneal-cavity washings. ●, Radioactivity; ○, NaCl concentration, determined by conductivity measurements. Cuts made between fractions containing heparin and fractions containing less highly sulphated glycosaminoglycans are indicated by vertical lines through the elution patterns. Two separate fractions containing heparin, labelled A and B, were recovered from lung (see the Results section).

out. It was washed between fractionations by repeating the entire gradient elution sequence.

Column fractions containing heparin with NA, LA and HA for antithrombin were combined according to the above protocol. NaCl concentrations were adjusted to 1.2 M, and pig mucosal heparin (2 mg) was added. Heparins were recovered by precipitation with ethanol.

RESULTS

Separation of ^{35}S-labelled heparins from other glycosaminoglycans on DEAE-Sephacel (see Fig. 1)

The elution patterns of the products from adipose tissue, heart and skin were similar. The heparin peak, eluted in approx. 1 M-NaCl, was quite well separated from a much larger earlier peak, comprising less highly sulphated connective-tissue glycosaminoglycans. With peritoneal washings the heparin peak was the major component, but again there was a distinct separation between two peaks. The elution pattern for the lung product was quite different: material was eluted at a position intermediate between the two peaks obtained from other sources. Therefore two lung products were worked up separately. The division into subfractions A and B is shown in Fig. 1: both were subsequently found to contain heparin.

Each heparin-containing fraction was finally purified by digestion with chondroitinase ABC. In every case material resistant to this treatment was extensively degraded by HNO_2 at pH 1.5. The elution pattern on Sephadex G-25 of each HNO_2-treated product was essentially identical with that of the well-characterized skin heparin. When the reaction with HNO_2 at pH 1.5 is allowed to proceed to completion, authentic heparins yield predominantly disaccharides, a small proportion of tetrasaccharides and virtually none of the larger products obtained from HNO_2-treated heparan sulphates (see, e.g., Gallagher & Walker, 1985). Thus, on the basis of their elution from DEAE-Sephacel at a higher NaCl concentration than other glycosaminoglycans, their resistance to chondroitinase ABC and degradation by HNO_2, the products were judged to be pure heparins.

In earlier work (Young & Horner, 1979), in which ^{35}S-labelled heparin proteoglycan from rat skin was incubated with a supernatant from homogenized rat small intestine, the addition of dextran sulphate greatly improved the subsequent recovery of ^{35}S-labelled heparin. Therefore dextran sulphate was again used as a carrier in the current experiments. However, in preliminary work, traces of the commercial preparation of dextran

Table 1. Weights and specific radioactivities of ^{35}S-labelled heparins recovered from various sources

The weights of heparin fractions recovered by gradient elution from DEAE-Sephacel, followed by digestion with chondroitinase ABC (for details, see the Experimental section) were determined colorimetrically with Azure A (Lam *et al.*, 1976). The specific-radioactivity values have been corrected to the date on which $H_2{}^{35}SO_4$ was administered.

Source of heparin	Weight of heparin (μg/rat)	Specific radioactivity (d.p.m./μg of heparin)
Adipose tissue	3.8	1136
Heart	5.5	551
Lungs (product A)	18.8	775
Lungs (product B)	8.3	809
Peritoneal cavity	35.7	8731
Skin	1388.8	841

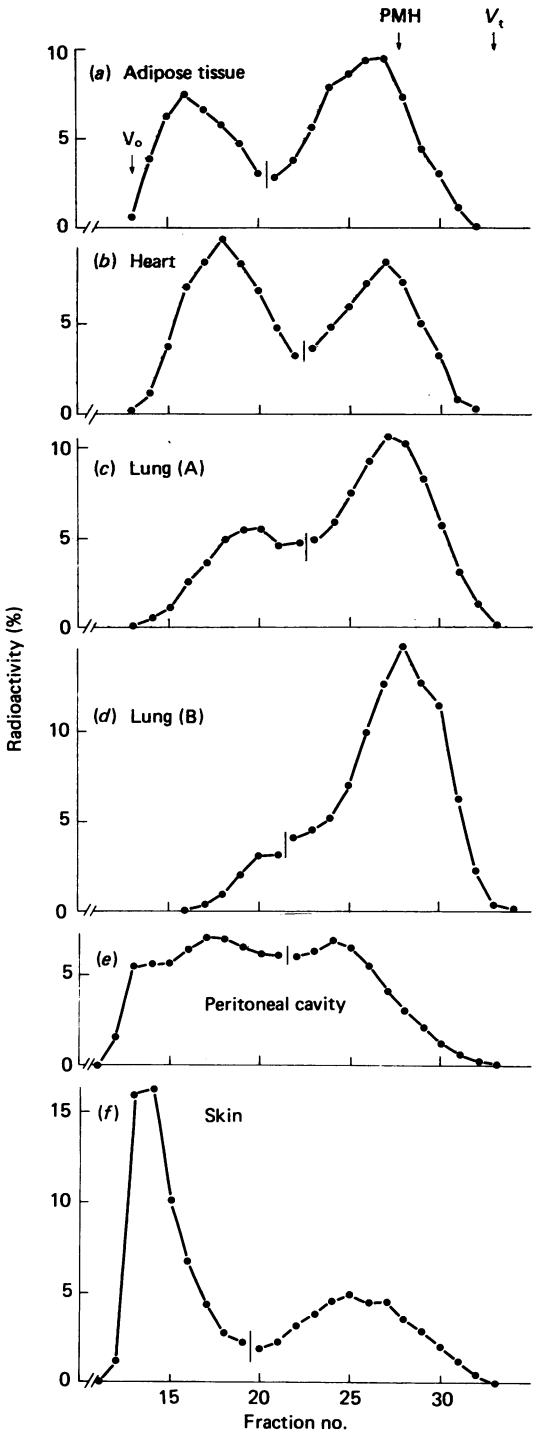

Fig. 2. Gel filtration on a column of Bio-Gel A-15m of pure ^{35}S-labelled heparins, after DEAE-Sephacel chromatography and treatment with chondroitinase ABC (see the Experimental section)

The six panels show fractionations of heparins from (*a*) adipose tissue, (*b*) heart, (*c*) lung (product A), (*d*) lung (product B), (*e*) peritoneal-cavity washings and (*f*) skin. The vertical line on each elution pattern indicates the division between each proteoglycan fraction and the corresponding depolymerization products fraction. The arrow labelled PMH marks the peak of the elution curve of commercial pig mucosal heparin on this column. The ordinates indicate percentages of total radioactivity in each sample applied to the column.

sulphate were carried through the purification and interfered with the metachromatic assay for heparin. This problem was eliminated by purifying the dextran sulphate as described.

The weights and specific radioactivities of the six heparin products are given in Table 1. Skin was first shown to be the predominant source of heparin in the rat by Monkhouse *et al.* (1957). In the current experiments skin accounted for 95% of the heparin recovered. The specific radioactivity of heparin from the peritoneal cavity was approximately one order of magnitude higher than that of heparin from any other source, presumably because $H_2{}^{35}SO_4$ was injected intraperitoneally.

In discussing the relative proportions of various heparin fractions, it has been assumed that different fractions from the same source had the same specific radioactivities. There was simply insufficient material to determine the mass of many of the products. However, in earlier work with ^{35}S-labelled heparins from rat skin (Horner & Young, 1982), ^{35}S was quite evenly distributed in proteoglycans, chains and depolymerization products.

Fractionation of pure heparins on Bio-Gel A-15m (see Fig. 2)

Heparins recovered after chondroitinase ABC digestion were gel-filtered on a column of Bio-Gel A-15m. Except with lung product B, two peaks were obtained. In most cases the separation was quite distinct. The peritoneal-cavity heparin gave an extended elution pattern: division into proteoglycan and depolymerization-product fractions was therefore made rather arbitrarily. Lung product B gave only one peak. The assignment of 90% of this heparin to the depolymerization-products fraction was again rather arbitrary.

Skin yielded the largest proteoglycans. Lungs contained the smallest proteoglycans and the smallest depolymerization products. There was also appreciable variation in the proportions of depolymerization products: skin containing the lowest proportion and lungs the highest.

Depolymerization of samples of the putative proteoglycan fractions with NaOH established their authenticity. A sample of each depolymerization-products fraction was also treated with alkali: no depolymerization was observed (results not shown).

Gel filtration of proteoglycan fractions on Bio-Gel A-50m (see Fig. 3)

As several of the proteoglycan peaks were eluted close to the V_0 of Bio-Gel A-15m, a sample of each was applied to a column of Bio-Gel A-50m. The skin proteoglycan, $M_r \sim 1 \times 10^6$ (Horner, 1971; Robinson *et al.*, 1978), is clearly appreciably larger, on average, than the other proteoglycans, although it overlaps appreciably with these, indicating considerable polydispersity. The relative sizes of proteoglycans from other sources were: adipose tissue > peritoneal cavity > hearts > lungs. They were all larger, on average, than chains from skin ($M_r \sim 80000$; Robinson *et al.*, 1978).

Fractionation of proteoglycan fractions on antithrombin–agarose (see Fig. 4)

There was an enormous range in the proportions of HA proteoglycans, from 59% to 9%. The relative proportions of the HA fractions were adipose tissue > heart > skin > lungs > peritoneal cavity.

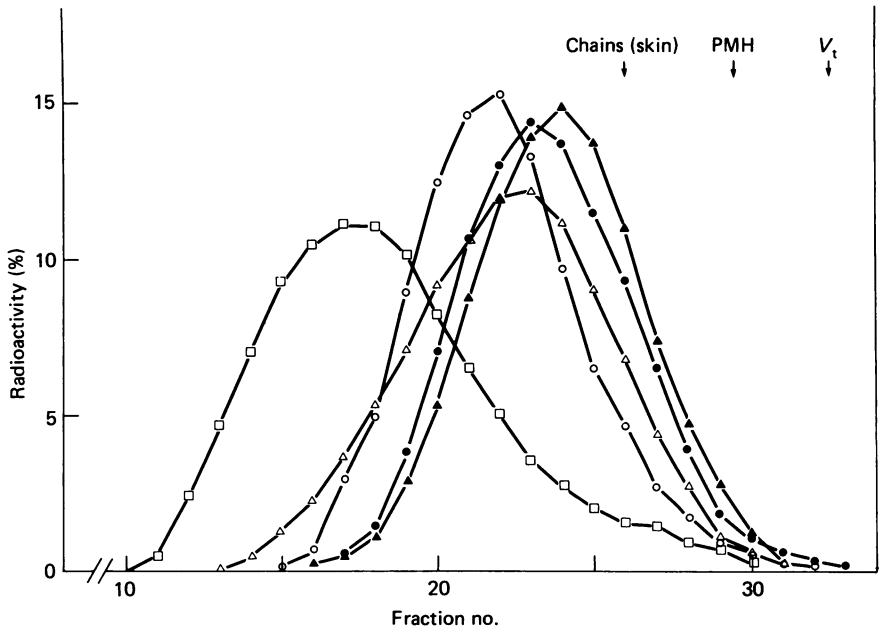

Fig. 3. Gel filtration on a column of Bio-Gel A-50m of ^{35}S-labelled heparin proteoglycan fractions

These products were obtained by gel filtration on a column of Bio-Gel A-15m (see Fig. 2). They were isolated from: ○, adipose tissue; ●, heart; ▲, lung (product A); △, peritoneal cavity; □, skin. The arrow labelled 'Chains (skin)' marks the peak of the elution curve for chains prepared from the skin proteoglycan. The arrow labelled PMH marks the peak of the elution curve for commercial pig mucosal heparin. The ordinate indicates percentages of the total radioactivity in each sample of proteoglycan. Further experimental details are given in the Experimental section.

Fractionation of chains on antithrombin–agarose (see Fig. 5)

In previous work with rat skin heparin, no NA chains were obtained. In the present work a trace (1%) was found in the skin, and higher proportions in all other sources. The proportion was highest in lung (41%). It was eluted as two distinct peaks of radioactivity, which were recovered separately. The peritoneal-cavity proteoglycan fraction also gave a high yield of NA chains (39%). The proportions of HA chains from different sources ranged from 28% to 4%. The relative proportions were adipose tissue > heart > skin > lungs > peritoneal cavity (the same sequence as that of the HA proteoglycans).

Fractionation of depolymerization products on antithrombin–agarose (see Fig. 6).

The proportions of HA depolymerization products from different sources varied from 13% to 6%, a much less dramatic range than the differences between proportions of HA proteoglycans or HA chains. The relative proportions of HA depolymerization products were: adipose tissue > lungs (product B) > heart > skin > lungs (product A) > peritoneal cavity.

Gel filtration of fractions recovered from antithrombin–agarose

Fractions separated by affinity chromatography were gel-filtered on various grades of Bio-Gel A. The data are summarized as $K_{av.}$ values in Table 2, which includes values for pig mucosal heparin for comparison. $K_{av.}$ values do not give a complete picture: Fig. 7 illustrates some of the varied elution patterns obtained. It shows the gel filtration of the peritoneal-cavity depolymerization products on Bio-Gel A-1.5m. This is one of the cases in which NA heparin was subdivided into fractions I_a and I_b. These products are very polydisperse, each showing overlapping peaks containing heparins which are both larger and smaller than pig mucosal heparin. Fraction I_a contains more extensively degraded material than does fraction I_b.

A review of these data reveals very few consistent features. LA and HA depolymerization products were always larger than pig mucosal heparin. HA fractions were usually slightly larger than cognate LA fractions. NA fractions varied greatly, being larger, smaller or equal in size to pig mucosal heparin, with a definite bimodal size distribution in adipose tissue, lung (product A) and peritoneal cavity.

The lung product-B fractions were equal in size to pig mucosal heparin or larger. This was surprising; in fact the decision to gel-filter on Bio-Gel A-0.5m was made on the assumption that they were smaller products. The alternative explanation for the 'early' elution of lung product B from DEAE-Sephacel is that it has a relatively low sulphate content. It is therefore noteworthy that this unusual product contained more HA material than did lung product A, which was presumably more highly sulphated.

In the case of chains, the NA fractions were consistently smaller than cognate LA and HA products and smaller than pig mucosal heparin. I_a fractions were smaller than I_b fractions. Size differences between LA and HA chains were small, and they were all larger than pig mucosal heparin. There was insufficient material from heart and adipose tissue to do gel filtrations.

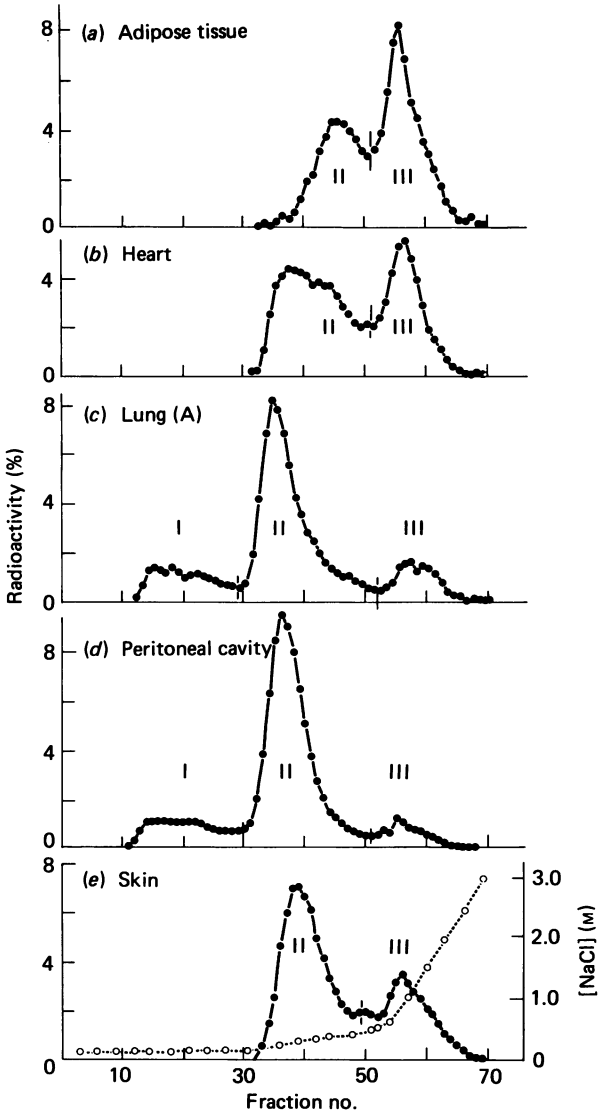

Fig. 4. Fractionations of ^{35}S-labelled heparin proteoglycans by elution in a NaCl concentration gradient from a column of antithrombin–agarose

The ^{35}S-labelled heparin proteoglycan fractions were from (*a*) adipose tissue, (*b*) heart, (*c*) lung (product A), (*d*) peritoneal cavity and (*e*) skin. The procedure yielded fractions with no affinity (I), low affinity (II) and high affinity (III) for antithrombin. Vertical lines drawn through the elution patterns indicate divisions made between these fractions. ●, Radioactivity; ○, NaCl concentration, determined by measuring conductivity. The same NaCl concentration gradient shown in the lowest panel was obtained reproducibly in each fractionation, by using an LKB Ultrograd gradient maker. The ordinates indicate percentages of the total radiaoctivity in each sample applied to the column. Further experimental details are given in the Experimental section.

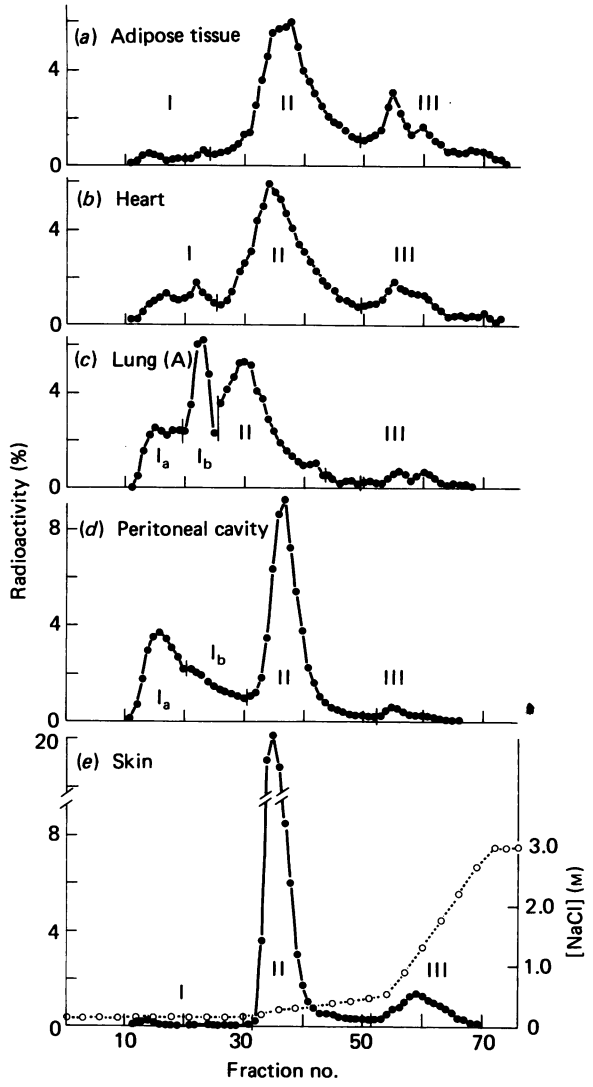

Fig. 5. Fractionations of ^{35}S-labelled heparin chains by elution in a NaCl concentration gradient from a column of antithrombin–agarose

The ^{35}S-labelled heparin chain products, prepared from the corresponding proteoglycan fractions by treatment with NaOH, were from (*a*) adipose tissue, (*b*) heart, (*c*) lung (product A), (*d*) peritoneal cavity and (*e*) skin. The procedure yielded fractions with no affinity (I), low affinity (II) and high affinity (III) for antithrombin. In (*c*) and (*d*) the 'no affinity' material was subdivided into fractions I_a and I_b as indicated. ●, Radioactivity; ○, NaCl concentration.

DISCUSSION

Heparin proteoglycans consist of polysaccharide chains linked to a unique polypeptide composed exclusively of serine and glycine in essentially equimolar proportions. The polysaccharide chains are released from the polypeptide by β-elimination with alkali (Robinson *et al.*, 1978). The term 'chains' is used here only in this sense; chains were derived from proteoglycans by treatment with alkali. In each heparin source studied, proteoglycans were the largest forms of heparin obtained by gel filtration. They were subsequently shown to undergo appreciable decrease in size when treated with alkali.

The term 'depolymerization products' has been used here as a general term for all heparins smaller than proteoglycans (from the same source). They were not decreased in size by treatment with alkali (this indicates that none of the heparin sources studied contained

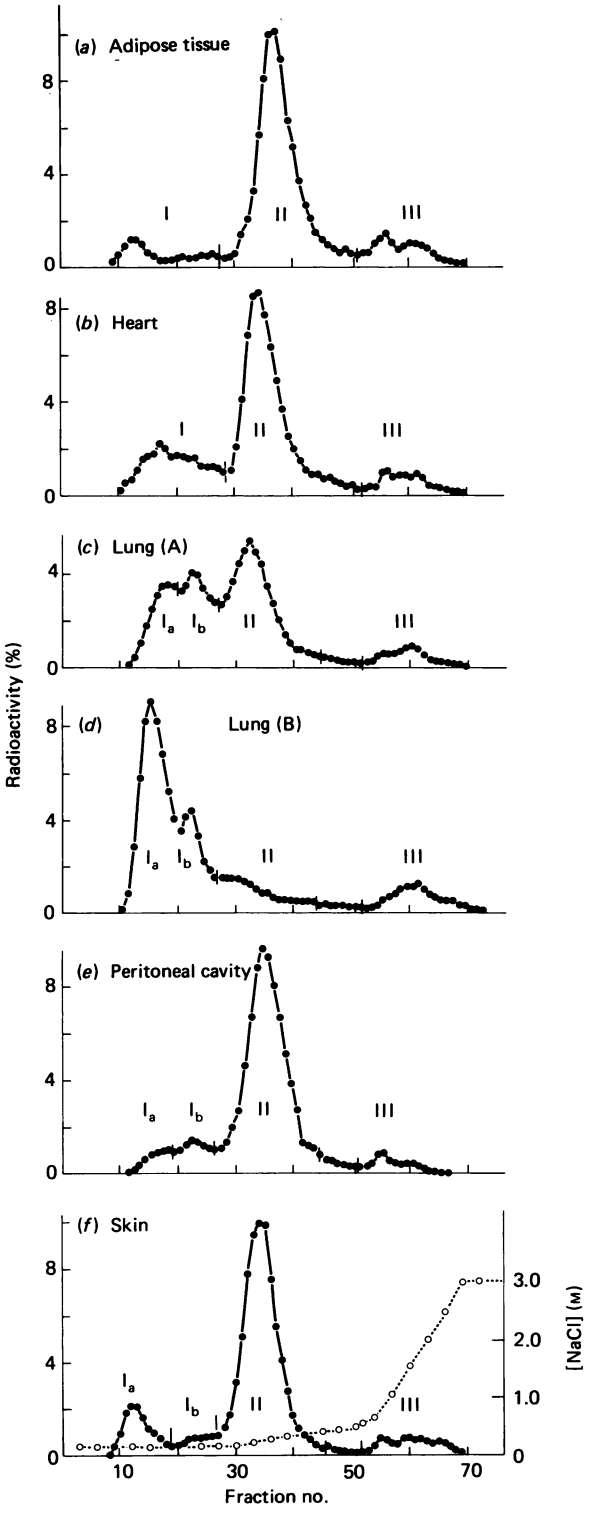

Fig. 6. Fractionations of ^{35}S-labelled heparin depolymerization products by elution in a NaCl concentration gradient from a column of antithrombin–agarose

The ^{35}S-labelled heparin depolymerization-product fractions were from (*a*) adipose tissue, (*b*) heart, (*c*) lung (product A), (*d*) lung (product B), (*e*) peritoneal cavity and (*f*) skin. The procedure yielded fractions with no affinity (I), low affinity (II) and high affinity (III) for antithrombin. In (*c*), (*d*), (*e*) and (*f*) the 'no affinity' material was subdivided into fractions I_a and I_b as indicated. ●, Radioactivity; ○, NaCl concentration.

distinct separable populations of large and small proteoglycans).

The arguments advanced in this discussion assume that heparins are invariably synthesized as proteoglycans (see the review by Björk & Lindahl, 1982) and that depolymerization occurs subsequently. Endoglycosidases, which degrade the polysaccharide chains of heparin proteoglycans, have been demonstrated in rat small intestine (Horner, 1972; Young & Horner, 1979), in human platelets (Thunberg *et al.*, 1982) and in mouse mastocytoma tissue (Ögren & Lindahl, 1975, 1976). Thunberg *et al.* (1982) have shown that there are at least two types of heparin endoglycosidase. The enzymes from human platelets and mouse mastocytoma have pH optima of 6.0 and 5.0 respectively. Both are endo-β-glucuronidases, but the platelet enzyme has a lower degree of substrate specificity than the mastocytoma enzyme. Only the platelet enzyme cleaves the β-glucuronidic linkage in heparin's antithrombin-binding sites, thereby destroying anticoagulant activity. This enzyme readily degrades commercial heparin and heparan sulphate, which are poor substrates for the mastocytomal enzyme.

The experiments described here showed no consistent pattern in the proportions of different types of heparin from different sources in the rat, in terms either of molecular size distribution or of affinities for antithrombin. Skin contained the largest proteoglycans and the highest proportion of proteoglycans. Lungs contained the smallest proteoglycans, the smallest depolymerization products and the highest proportion of depolymerization products. Adipose-tissue proteoglycans, chains and depolymerization products contained higher proportions of HA material than the corresponding products from other sources. The corresponding peritoneal-cavity products had the lowest proportions of HA material in each case. The biosynthesis of HA binding sites involves post-polymerization modifications to polysaccharide chains (Björk & Lindahl, 1982). The structure of these sites has been elucidated (Lindahl *et al.*, 1983), but the mechanisms controlling their synthesis are still not fully understood (Horner & Young, 1982; Jacobsson *et al.*, 1985).

When heparin proteoglycans are depolymerized, either chemically to produce chains or enzymically to produce depolymerization products, the proportion of HA material should decrease as heparins containing HA binding sites become separable from heparins with NA or LA to which they were previously bound covalently. This effect was observed in the earlier work with rat skin heparins (Horner & Young, 1982). The pattern was clearly followed in the products from adipose tissue, heart and skin and to a lesser degree in lung. This molecular-size-dependent trend was very unimpressive for peritoneal-cavity products. The proteoglycan fraction only contained 9% HA material: depolymerization products contained 6% HA material. This suggests that very few HA binding sites are ever synthesized in the peritoneal-mast-cell proteoglycan.

The data for peritoneal-cavity heparins were particularly interesting. In cases where rat peritoneal mast cells have been purified from peritoneal washings by density-gradient centrifugation (Horner, 1977; Yurt *et al.*, 1977*a*), the heparins subsequently isolated were exclusively large proteoglycans. It has also been shown that purified mast cells can release granule-associated heparin

Table 2. Gel filtration of rat ^{35}S-labelled heparins, previously fractionated on antithrombin–agarose, and of commercial pig mucosal heparin, on columns of Bio-Gels A-15m, A-1.5m and A-0.5m

These data indicate the relative sizes of some of the heparin fractions obtained in this study, to illustrate their diversity. Three porosity grades of Bio-Gel A were used. A sample of commercial pig mucosal heparin was run through each column for comparison. Examples of the gel filtrations from which these data were calculated are shown in Fig. 7.

Depolymerization products from:	$K_{av.}$* values						
	I_a	I_b	I	II	III	Pig mucosal heparin	Bio-Gel used
Lung (B)	0.30	0.30	—	0.20	0.20	0.30	A-0.5m
Adipose tissue	—	—	0.25, 0.70	0.35	0.25	0.50	A-1.5m
Heart	—	—	0.50, 0.65	0.35	0.30		
Lung (A)	0.70	0.55	—	0.40	0.35		
Peritoneal cavity	0.30, 0.75	0.35, 0.65	—	0.25	0.20–0.25		
Skin	—	—	0.70	0.55	0.50	0.75	A-15m
Chains from:							
Lung (A)	0.75	0.55	—	0.40	0.35	0.50	A-1.5m
Peritoneal cavity	0.65	0.55	—	0.30	0.25		
Skin	—	—	—	0.45	0.45	0.75	A-15m

* As defined by Laurent & Killander (1961).

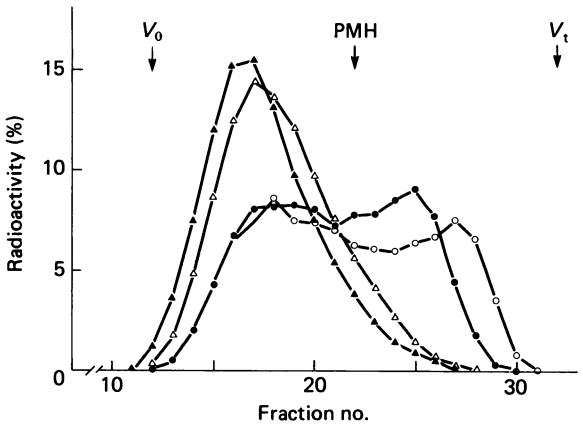

Fig. 7. Gel filtration of four ^{35}S-labelled heparin depolymerization-product fractions from peritoneal washings, applied separately to a column of Bio-Gel A-1.5m

These heparins had been recovered after separation by affinity chromatography, on a column of antithrombin–agarose (see Fig. 6*e*), into fraction I_a, with no affinity (○), fraction I_b, with no affinity (●), fraction II, with low affinity (△) and fraction III, with high affinity for antithrombin (▲). The arrow marked PMH marks the peak elution position of commercial pig mucosal heparin.

proteoglycan without any depolymerization occurring (Yurt *et al.*, 1977*b*). In the present experiments heparin recovered from peritoneal washings contained a high proportion of depolymerization products. These may not have been associated with mast cells. The physiological fate of released heparin is unknown, but experimental evidence strongly implicates macrophages in heparin catabolism. Cultured mouse peritoneal macrophages can ingest mast-cell granules containing ^{35}S-labelled heparin and release [^{35}S]sulphate (Lindahl *et al.*, 1979). They have receptors on their plasma membranes for commercial heparin (Bleiberg *et al.*, 1983), which they take up and desulphate (Fabian *et al.*, 1978). As macrophages are the major cell type in the rat peritoneal cavity (Pratten *et al.*, 1977), there is strong evidence *in vitro* to implicate peritoneal macrophages in peritoneal-mast-cell heparin proteoglycan catabolism *in vivo*.

The depolymerization-product fractions showed great diversity (see the Results section). Some sources contained NA heparin that was eluted from antithrombin–agarose as distinct separable subfractions. When feasible, these were recovered separately. The earlier-eluted subfractions contained more smaller material, but the differences were not dramatic. The structural differences between NA heparins and LA heparins are not known, but it appears that factors other than relative size are involved.

Only skin, lungs and the peritoneal cavity yielded enough chains for size comparison after fractionation on antithrombin–agarose. They varied greatly in size, NA chains being appreciably smaller than the others. These were found in all sources, though there was only a trace in skin.

NA proteoglycans were obtained only from lungs and the peritoneal cavity. Proteoglycans from sources other than skin and adipose tissue were not much larger than chains from the skin proteoglycan, and their constituent chains were much smaller, being included in Bio-Gel A-1.5m.

It is impossible to say whether mast cells in all the heparin sources examined initially synthesized proteoglycans as large as those produced by dermal mast cells. However, the data clearly indicate that there were large differences in endoglycosidase activities in different tissues. Heparin catabolism occurred most extensively in lungs. This high activity was also characterized by a high proportion of NA depolymerization products.

Further studies may reveal the presence of unique heparin endoglycosidases in different cells and tissues. It is equally plausible to postulate that mast cells in

different sites produce unique variants of heparin. Also, tissue differences in the activities of exoenzymes, which act on the products of endoglycosidase activity (Dorfman & Matalon, 1976), may affect overall rates of heparin catabolism. The products of exoenzyme activity would not be precipitated by CPC in 1.0 M-NaCl and would therefore have been discarded in the present experiments.

This study may quite reasonably be said to have raised more questions than it has answered. It does, however, clearly indicate that one cannot generalize when discussing heparin metabolism. Each heparin source appears to be unique, with respect to the proteoglycan which it synthesizes and to the manner in which this product is subsequently depolymerized.

I thank Dr. M. Wickerhauser for the gift of human antithrombin, Mrs. G. Hopkins and Mrs. M. Robinson for technical assistance and Miss J. Bilyk for secretarial help. This work was supported by the Heart and Stroke Foundation of Ontario.

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Received 25 February 1986/9 June 1986; accepted 24 July 1986